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ORIGINAL RESEARCH

Native Small Airways Secrete Bicarbonate

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Abstract

Since the discovery of Cl^- impermeability in cystic fibrosis (CF) and the cloning of the responsible channel, CF pathology has been widely attributed to a defect in epithelial Cl^- transport. However, loss of bicarbonate (HCO_3^-) transport also plays a major, possibly more critical role in CF pathogenesis. Even though HCO_3^- transport is severely affected in the native pancreas, liver, and intestines in CF, we know very little about HCO_3^- secretion in small airways, the principle site of morbidity in CF. We used a novel, mini-Ussing chamber system to investigate the properties of HCO_3^- transport in native porcine small airways ($\sim$1 mm ϕ). We assayed HCO_3^- transport across small airway epithelia as reflected by the transepithelial voltage, conductance, and equivalent short-circuit current with bilateral 25-mM HCO_3^- plus 125-mM NaGlu Ringer's solution in the presence of luminal amiloride (10 μM). Under these conditions, because no major transportable anions other than HCO_3^- were present, we took the equivalent short-circuit current to be a direct measure of active HCO_3^- secretion. Applying selective agonists and inhibitors, we show constitutive HCO_3^- secretion in small airways, which can be stimulated significantly by β-adrenergic– (cAMP) and purinergic (Ca^{2+}) -mediated agonists, independently. These results indicate that two separate components for HCO_3^- secretion, likely via CFTR- and calcium-activated chloride channel–dependent processes, are physiologically regulated for likely roles in mucus clearance and antimicrobial innate defenses of small airways.

Keywords: electrolyte transport; airway function; cystic fibrosis transmembrane conductance regulator; airway surface liquid; mucus

Clinical Relevance

The authors describe a previously unknown basic physiological function of normal small respiratory airways; that is, the active secretion of bicarbonate (HCO_3^-) into airway surface liquids. Furthermore, they demonstrate that secretion is not only constitutive, but can be enhanced independently by either cystic fibrosis (CF) transmembrane conductance regulator (CFTR)/cAMP–dependent or calcium-activated chloride channel/Ca^{++}–dependent HCO_3^- secretory pathways. This discovery not only adds new functions to the description of basic normal physiology of the lung, but also provides the first physiological evidence in native small airway epithelia to link the generalized defect in HCO_3^- transport in CF to the most vulnerable region of respiratory pathogenesis.

Even though cystic fibrosis (CF) transmembrane conductance regulator (CFTR) mutations are well recognized for disrupting Cl^- transport, major pathologies associated with CF are closely associated with defective bicarbonate (HCO_3^-) transport (1–10). The destruction of the pancreas forces a focus on abnormal HCO_3^- transport in CF pathology (2, 3, 11). Very recent evidence indicates that loss of HCO_3^- secretion leads to abnormal mucus maturation and poor release into the lumens of the gut (12, 13) and the reproductive tract (14), which may be at play in other airway diseases as well. These results specify a close link between abnormal HCO_3^- transport and the characteristically thickened mucus in multiple organ systems that likely include the small airways (15). In fact, abnormally thick mucus formation (mucoviscidosis) with consequent obstruction of small airways is the foremost cause of morbidity and mortality in patients with CF (16–18). Although suspected for many years (1, 19–21), and despite this apparent nexus between abnormal HCO_3^- secretion and

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abnormal mucus, whether HCO_3^- transport occurs in native small airways is not yet known.

Until very recently, significant technical hurdles have precluded direct physiological investigation of the miniscule and fragile structures of native small airways. We have now overcome several of these limitations with a new system to evaluate electrogenic responses of ion transport in native small airways of roughly 1 mm diameter (22) using pigs as models for function. The term "small airway" may carry different implications in different contexts. To clarify our use herein, we note that, in adult humans, bronchi are typically defined as ciliated airways of more than 2 mm internal diameter that extend from the trachea to approximate generations 7–9 in the bronchial tree, are characterized by rings of cartilage to prevent collapse, and are associated with abundant submucosal glands. By strict definition, bronchioles (< 2 mm diameter) lack cartilaginous rings as well as submucosal glands, are collapsible, also ciliated, and extend beyond bronchi to generations 15–19 (23, 24). These airways are believed to be important in inflammation in early chronic obstructive pulmonary disease (24), asthma (25), and CF (26).

In our young pigs, we cannot strictly classify our roughly 1-mm-diameter specimens as bronchioles, because plaques of cartilage and some gland acini are present, but neither do they qualify as bronchi, because they lack both continuous rings and significant submucosal glands. Thus, because they are small, and are most likely to at least be from the zone of transition to bronchioles in these young pigs, we use the term "small airway" to refer to the airways used in these studies. We surmise that the properties of our preparations reflect those of the mucosal epithelium, because most maneuvers were performed on the mucosal surface, avoiding direct exposure to any functional gland parenchyma that might be present. Furthermore, contributions from glands would require gland duct openings, which would likely shunt the high diffusion potentials that we observed. From the histological similarity of the mucosal epithelia in our preparations with that of smaller, true bronchioles, we surmise that major differences in electrolyte transport properties with bronchioles are unlikely, but conceivably possible.

Welsh and Smith (27) and Coakley and colleagues (28) reported that HCO_3^- secretion occurs in normal, but not in CF, cultured bronchial airway cells. However, HCO_3^- secretion has never been demonstrated in native, intact small airway epithelium until now. We examined freshly excised, native porcine small airways as a model of human tissue to determine the presence and physiological responses of HCO_3^- secretion. We found constitutive HCO_3^- secretion in small airways that is stimulated significantly by cAMP- and Ca^{2+}-mediated agonists, and respectively inhibited by selective antagonists, to suggest two pathways of HCO_3^- secretion, possibly via CFTR- and calcium-activated chloride channels (CaCCs)–dependent transport.

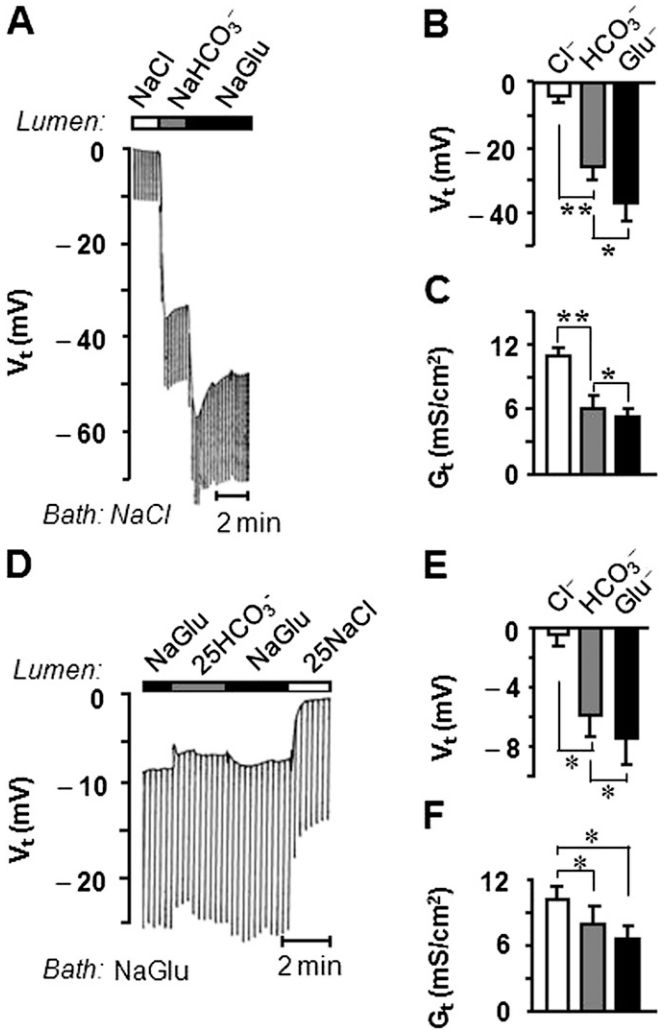

Figure 1. Anion selectivity of small airways. Small airways show significant conductance to Cl^- and bicarbonate (HCO_3^-), as indicated by changes in transepithelial potential (V_t) and transepithelial conductance (G_t) after anion substitution with 150 mM gluconate in the presence of 3-isobutyl-1-methylxanthine (IBMX)/forskolin (Fsk) and amiloride in the luminal bath. Representative electrical trace (*A*) of V_t responses to substitutions of 150 mM gluconate (*black*) on the lumen of a small airway with equimolar HCO_3^- (*gray*) or Cl^- (*white*) while maintaining 150 mM NaCl on the serosal surface. Summary of results of anion replacements on V_t (*B*) and G_t (*C*). Representative trace (*D*) of constitutive electrical properties in bilateral 150 mM NaGlu with responses to substitution of 25 mM luminal Glu with either equimolar Cl^- or HCO_3^-, which significantly depolarized the electrogenic Na^+-dependent component of V_t. Note that Cl^--induced depolarization was more than three times that due to HCO_3^-, indicating that HCO_3^- conductance is significantly greater than the impermeant gluconate, but much less than Cl^- in shunting the Na^+ absorptive potential. Summary of V_t (*E*) and G_t (*F*) responses to replacements of 25 mM Glu^- (*black*) with equimolar HCO_3^- (*gray*) or Cl^- (*white*). $25HCO_3^-$, 25 mM $NaHCO_3^-$; 25NaCl, 25 mM NaCl. $^*P < 0.05$, $^{**}P < 0.001$; data presented are means ± SEM.

Table 1: Constitutive and Agonist-Induced Transepithelial Electrical Properties of Small Airways

	Agonists	V_t (*mV*)	G_t (*mS/cm²*)	I_{sc}^{eq} (*μA/cm²*)
Constitutive				
125 mM NaCl + 25 mM $NaHCO_3$	No agonists (*n* = 4)	−2.3 ± 1.3	14.2 ± 3.8	22.1 ± 9.8
125 mM NaGlu + 25 mM $NaHCO_3$	No agonists (*n* = 24)	−1.2 ± 0.3	7.7 ± 0.8	10.6 ± 1.4
		ΔV_t (*mV*)	ΔG_t (*mS/cm²*)	ΔI_{sc}^{eq} (*μA/cm²*)
Mediators				
cAMP	Fsk/IBMX (*n* = 16)	1.7 ± 0.3	1.0 ± 0.2	15.0 ± 1.6
	IPR (*n* = 4)	1.3 ± 0.4	0.4 ± 0.2	10.2 ± 1.2
Ca^{2+}	UTP (*n* = 10)	0.6 ± 0.1	0.9 ± 0.2	7.8 ± 1.1
cAMP + Ca^{2+}	PGE2 (*n* = 4)	1.3 ± 0.3	0.6 ± 0.1	12.6 ± 4.4
	Fsk/IBMX + UTP (*n* = 5)	2.2 ± 0.6	1.9 ± 0.6	21.9 ± 4.4

Definition of abbreviations: Δ, induced change from paired constitutive values; Fsk, forskolin; Glu, gluconate; G_t, transepithelial conductance; IBMX, 3-isobutyl-1-methylxanthine; IPR, isoproterenol; I_{sc}^{eq}, equivalent short-circuit current; PGE2, prostaglandin E2; V_t, transepithelial potential. Fsk (10 μM) plus IBMX (100 μM) and β-agonist, IPR (10 μM), were used as cAMP-mediated agonists. UTP (100 μM) was considered as an essentially Ca^{2+}-mediated P2Y2 agonist, and PGE2 (1 μM) appeared to have properties of both a Ca^{2+}-mediated and cAMP-mediated agonist. $NaHCO_3^-$ (25 mM) and NaGlu (125 mM) Ringer's solutions were present bilaterally, and luminal amiloride (10 μM) was present to inhibit epithelial Na^+ channel–dependent Na^+ transport in all conditions. Constitutive values: V_t, G_t, and I_{sc}^{eq}. Induced changes from paired constitutive values: ΔV_t, ΔG_t, and ΔI_{sc}^{eq} (*n* = number of measurements in as many tissues; values are means ± SE).

Materials and Methods

Tissue Procurement and Preservation

Intact lungs were collected from farm pigs (30–40 kg) of either sex immediately after being killed by stunning, followed by immediate exsanguination to reduce pulmonary hemorrhage. Excised lungs were placed in plastic bags under crushed ice (< 10°C) until being used (22). All specimens reported were examined within 8 hours of death. The Institutional Animal Care and Use Committee of the University of California San Diego approved the procedures used in this study.

Tissue Dissection and Electrophysiological Measurements

To minimize endogenous prostaglandins during dissection, indomethacin (1 μM) was present in Ringer's solution until mounting the tissue. Approximately 4 mm^2 of the opened and "flattened" airway was mounted with the serosal surface supported by a "trampoline" of an open weave polycarbonate mesh (400-μm openings; salvaged from tea bags) in a custom-fabricated mini-Ussing chamber (22). An area (~ 1 mm^2) of the mucosal surface was gently sealed under the polished end of a small glass capillary (radius ~ 0.6 mm). Both the apical and serosal sides of the tissue were perfused with defined solutions at roughly 37°C. The measurement of transepithelial potential (V_t), calculations of transepithelial conductance (G_t), and equivalent short-circuit current (I_{sc}^{eq}) have previously been described in detail (22). Briefly, open-circuit V_t was measured continuously with a high-impedance voltmeter; G_t was calculated from Ohm's law based on the deflections in V_t during

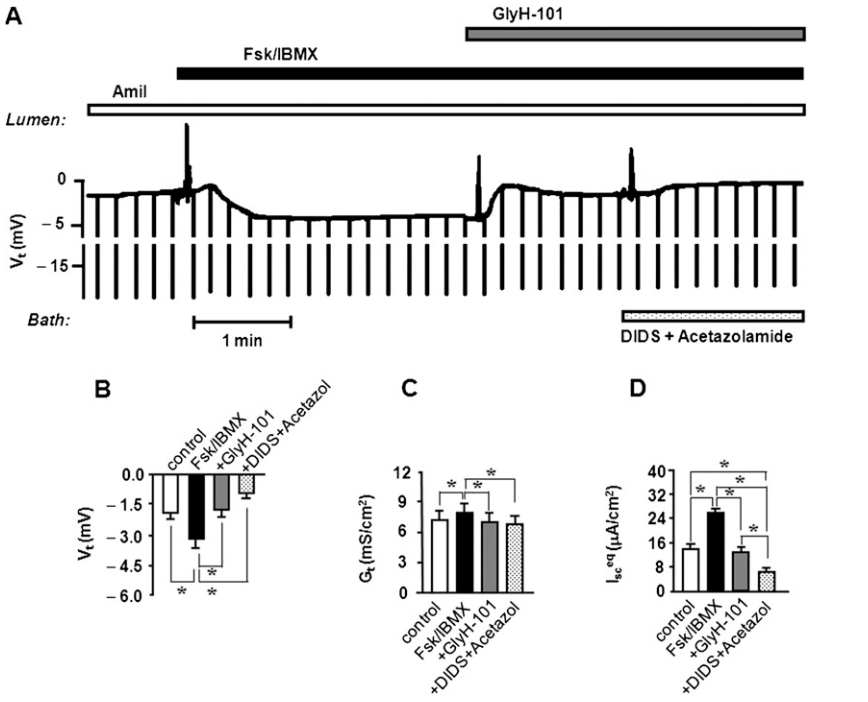

Figure 2. Effect of cAMP agonist Fsk/IBMX and inhibitors on HCO_3^- transport. (*A*) Representative trace of electrogenic HCO_3^- transport properties in native small airways with 25 mM HCO_3^- in both basolateral and luminal baths (Cl^- free). In the presence of luminal amiloride (Amil), mucosal addition of Fsk/IBMX significantly hyperpolarized V_t and stimulated equivalent short-circuit current (I_{sc}^{eq}). Luminal addition of cystic fibrosis (CF) transmembrane conductance regulator (CFTR) inhibitor, GlyH-101 blocked the I_{sc}^{eq} response. Furthermore, addition of serosal 4,4′diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) plus acetazolamide reduced the I_{sc}^{eq} to significantly less than the initial, unstimulated constitutive current (*white*). Summary of the effects of Fsk/IBMX (*black*), GlyH-101 (*gray*), and DIDS + acetazolamide (*stippled*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*). *$P < 0.05$; data presented are means ± SEM.

brief (1 s) constant current pulses (0.3–1.0 μA) passed intermittently (0.1 Hz) across the tissue. I_{sc}^{eq} was calculated from G_t and V_t using Ohm's law. As before (22), because a 150-mM Cl^- gradient in well preserved preparations of tissue generally generates approximately 40 mV (lumen negative), we discarded preparations that did not generate a V_t more negative than −25 mV with this gradient.

Solutions and Drugs

Ringer's solution contained Na^+ (150 mM), K^+ (4.6 mM), Mg^{2+}(1.0 mM), Ca^{2+}(1.0 mM), PO_4^{3-} (2.5 mM), Cl^- (150 mM), SO_4^{2+} (1.0 mM), acetate (2.0 mM), and glucose (10 mM), buffered to pH 7.4 with HCl. For anion diffusion studies, 150 mM NaCl was replaced with equimolar Na^+ gluconate (150-mM Cl^- gradients). For $NaHCO_3^-$ Ringer's solution, 25 mM $NaHCO_3^-$ plus 125 Na^+ gluconate were substituted for Cl^- as indicated. All HCO_3^- solutions were adjusted to pH 7.4 by gassing to equilibrium with 95% O_2 plus 5% CO_2.

3-Isobutyl-1-methylxanthine (IBMX; 100 μM), forskolin (Fsk; 10 μM), prostaglandin E2 (PGE2; 1 μM), isoproterenol (IPR; 10 μM), UTP (100 μM), ATP (100 μM), amiloride (10 μM), indomethacin (1 μM), 4,4′diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; 100 μM), acetazolamide (100 μM), 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS; 100 μM), niflumic acid (NFA; 100 μM), mecholyl (10 μM), substance P (1.0 μM), and vasoactive intestinal peptide (0.1 μM) were obtained from Sigma Chemical Co. (St. Louis, MO) and applied at the concentrations indicated in parentheses. GlyH-101 (50 μM), used as a CFTR inhibitor, was a generous gift from Dr. A. Verkman (University of California San Francisco).

HCO_3^- Transport Assay

All assays of electrogenic HCO_3^- transport were performed in bilateral 25-mM HCO_3^- plus 125-mM NaGlu Ringer's solution in the presence of luminal amiloride to block electrogenic Na^+ absorption and prevent Cl^- secretion. Because no major, transportable ion other than HCO_3^- was present, the changes in V_t, G_t, and I_{sc}^{eq} after stimulation were interpreted as due to changes in HCO_3^- secretion activity. The fact that known HCO_3^- transport inhibitors almost completely blocked this I_{sc}^{eq} supported this interpretation.

Statistical Analysis

The data are presented as means (± SE), and "*n*" is the number of tissues. Statistical significance was determined on the basis of Student's *t* test for paired samples. A *P* value less than 0.05 was taken as indicating a significant difference.

Results

HCO_3^- Conductance

We determined the apparent permeability of HCO_3^- relative to Cl^- and the impermeant anion, gluconate, in the presence of amiloride. After stimulation with Fsk plus IBMX, changes in V_t on replacing 150 mM Cl^- in the apical bathing solution with 150 mM HCO_3^- or 150 mM gluconate (Figures 1A and 1B) indicated that the native airway epithelium is approximately 1/5 as permeable to HCO_3^- as to Cl^-, as calculated from the Goldman equation (29). The value approximates the relative conductances reported previously for the CFTR channels in other preparations (6, 30). Likewise, the ratio of Cl^- and HCO_3^- conductances measured here generated a similar ratio (Figure 1C). We reduced the concentration of both HCO_3^- and Cl^- to the physiological concentration of 25 mM and repeated the substitutions. These maneuvers (without amiloride) resulted in shunting the constitutive V_t, most likely due electrogenic absorption of Na^+ in the absence of permeable anions. The fact that Cl^- caused relatively greater changes in V_t and G_t not only reveals the inherently high G_t to Cl^- compared with HCO_3^-(Figures 1D–1F), but also suggests that Cl^- is the more prevalent co-ion in electrogenic fluid absorption (22).

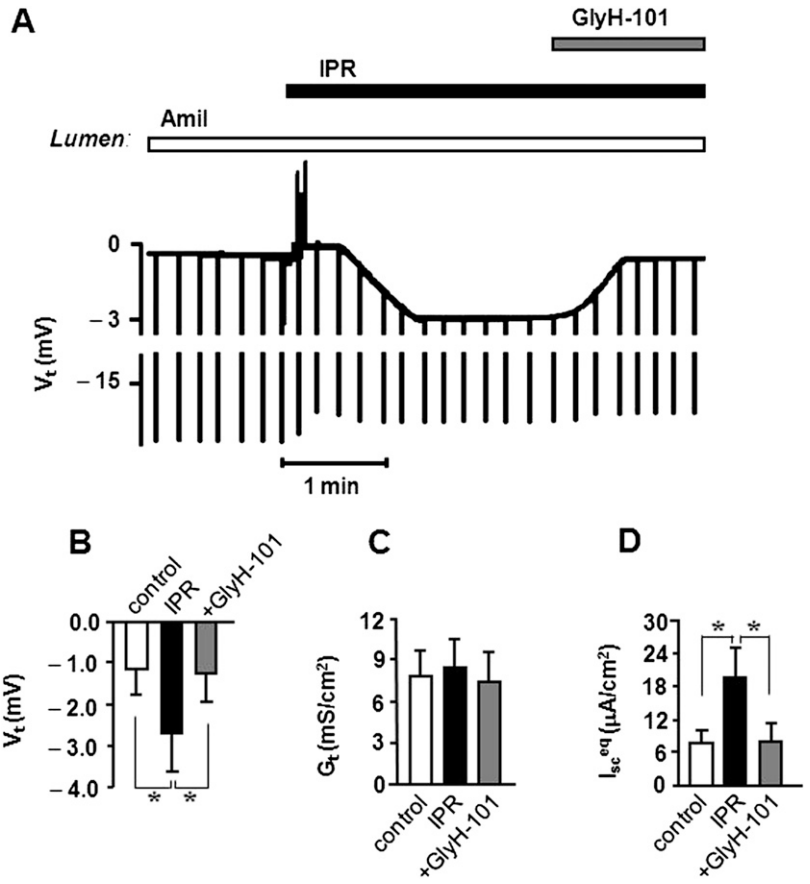

Figure 3. Effects of inhibitor GlyH-101on isoproterenol (IPR) stimulation of HCO_3^- transport. (*A*) Representative trace of changes in electrogenic HCO_3^- transport properties with addition of IPR (10 μM), which significantly hyperpolarized V_t and stimulated I_{sc}^{eq} ($P < 0.05$; $n = 3$). Luminal addition of inhibitor, GlyH-101, blocked stimulated HCO_3^- secretion. Summary constitutive properties (*white*) and of the effects of IPR (*black*) and GlyH-101 (*gray*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*). *$P < 0.05$; data presented are means ± SEM.

cAMP-Mediated HCO_3^- Secretion

Effects of cAMP agonists and CFTR inhibitor Gly-H 101. We first tested the effect of removing Cl^- from the media, after which approximately 50% of the constitutive I_{sc}^{eq} remained (Table 1). To show that HCO_3^- secretion is responsive to stimulation, and therefore likely to be a physiologically regulated function, we tested different agonists for effects on HCO_3^- I_{sc}^{eq} by elevating intracellular cAMP. Adding membrane-permeable Fsk/IBMX to the lumen (Figure 2) to elevate intracellular cAMP directly or adding the cAMP-mediated β-adrenergic agonist, IPR (Figure 3), to the bath solution significantly increased V_t, G_t, and I_{sc}^{eq} over constitutive values, indicating activation of electrogenic HCO_3^- secretion (i.e., I_{sc}^{eq} more than doubled). The CFTR inhibitor, GlyH-101 (22, 31, 32), in the lumen completely inhibited the cAMP-stimulated response and reduced the I_{sc}^{eq} to constitutive (unstimulated) levels (Figures 2 and 3; Table 1). Adding DIDS and acetazolamide basolaterally after the luminal inhibition with GlyH-101 to Fsk/IBMX–stimulated airways to inhibit any remaining HCO_3^--dependent current further reduced I_{sc}^{eq} to values that were approximately 50% of the constitutive values (Figure 2).

Applying basolateral DNDS (Figure 4) to inhibit HCO_3^- entry into the cells via basolateral sodium bicarbonate co-transporter and/or Cl^-/HCO_3^- transporters also inhibited approximately 80% of the stimulated response, and adding luminal GlyH-101 to inhibit CFTR again reduced I_{sc}^{eq} to approximately 50% of the constitutive values (Figure 4). We cannot account for the residual current after applying these inhibitors (which were also in the presence of amiloride and absence of Cl^-).

Effects of PGE2, GlyH-101, and NFA. We applied the secretagogue, PGE2, to the luminal solution. PGE2 increased I_{sc}^{eq} to more than twice the constitutive current, but GlyH-101 did not completely inhibit the stimulated increase. Adding the putative Ca^{2+} channel inhibitor, NFA, reduced the I_{sc}^{eq} to approximately 50% of the constitutive values (Figure 5).

Ca^{2+}-Mediated HCO_3^- Secretion

Effects of UTP and inhibitor NFA. We investigated the possibility that the Ca^{2+}-activated P2Y2 purinergic receptor ligand, UTP, might account for the GlyH-101 (possibly CFTR) –independent HCO_3^- secretion. In bilateral 25 mM $NaHCO_3^-$, UTP also significantly stimulated HCO_3^- secretion (Figure 6). Under these conditions, V_t hyperpolarized as G_t and I_{sc}^{eq} significantly increased, whereas all three responses were inhibited by addition of NFA, which decreased I_{sc}^{eq} significantly below the initial spontaneous current (Figure 6).

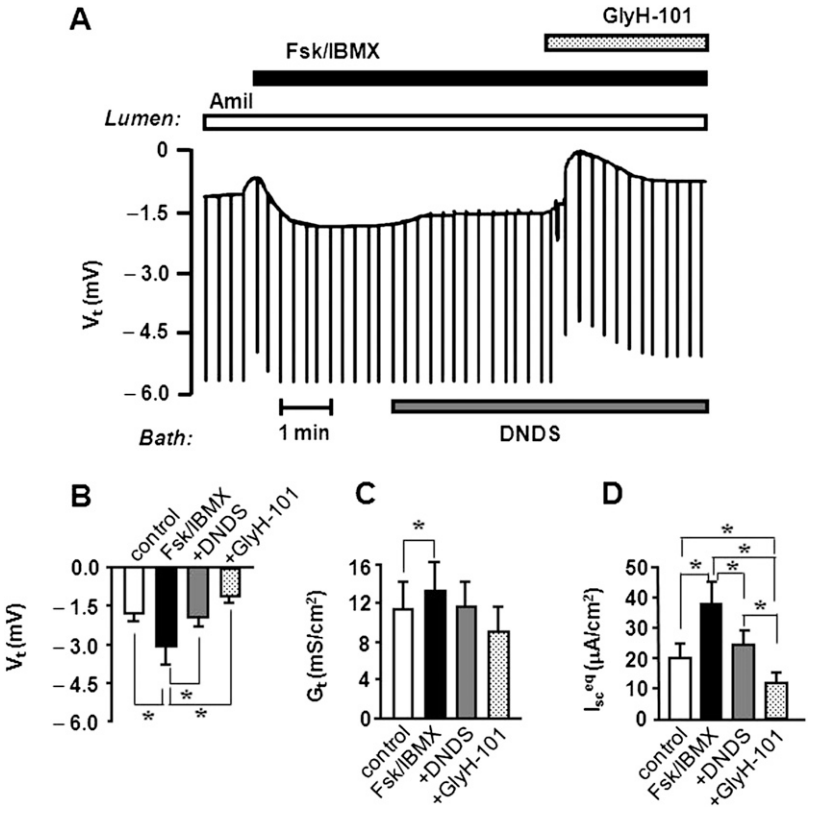

Figure 4. Effect of the inhibitors 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS) and GlyH-101 on Fsk/IBMX stimulation. (*A*) Representative trace of electrogenic HCO_3^- transport properties after luminal addition of Fsk/IBMX (*black*), which significantly hyperpolarized V_t, increased G_t, and stimulated I_{sc}^{eq} in the continuous presence of luminal amiloride (*white*). Subsequent contraluminal addition of the inhibitor DNDS (*gray*) significantly blocked V_t, G_t, and I_{sc}^{eq} response ($P < 0.05$; $n = 3$). Further luminal addition of GlyH-101 (*stippled*) reduced the remaining I_{sc}^{eq} to significantly less than the initial constitutive current. Summary of the effects of Fsk/IBMX (*black*), DNDS (*gray*), and GlyH-101 (*stippled*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*). *$P < 0.05$; data presented are means ± SEM.

Additive Effects of cAMP- and Ca^{2+}-Mediated Stimulation on HCO_3^- Secretion

We investigated potential additive effects of the cAMP-mediated agonist, Fsk/IBMX, combined with the Ca^{2+}-mediated agonist, UTP. We stimulated with Fsk and IBMX. and V_t, G_t, and I_{sc}^{eq} significantly increased ($P < 0.05$; $n = 5$). We then added luminal UTP, which further increased V_t, G_t, and I_{sc}^{eq} (Figure 7; Table 1). The additive responses were independent of the sequence of adding the agonist (data not shown).

Specificity of pathway inhibition. As a final test of independence of pathways, we stimulated with Fsk/IBMX and then tested inhibition of the response by adding NFA. The inhibitor had no discernible effect (Fsk/IBMX I_{sc}^{eq} = 25.0 ± 8.4 uA/cm^2 versus Fsk/IBMX + NFA I_{sc}^{eq} = 24.2 ± 8.5; $P > 0.08$; $n = 4$). Likewise, we stimulated with UTP and then inhibited with Gly-H-101. Similarly, the CFTR inhibitor did not block the UTP response (UTP I_{sc}^{eq} = 28.2 ± 3.8 versus UTP I_{sc}^{eq} + Gly-H-101 = 28.1 ± 3.9; $P > 0.42$; $n = 3$).

Other Agonists

We also tested for the effects of other agonists, including mecholyl, substance P,

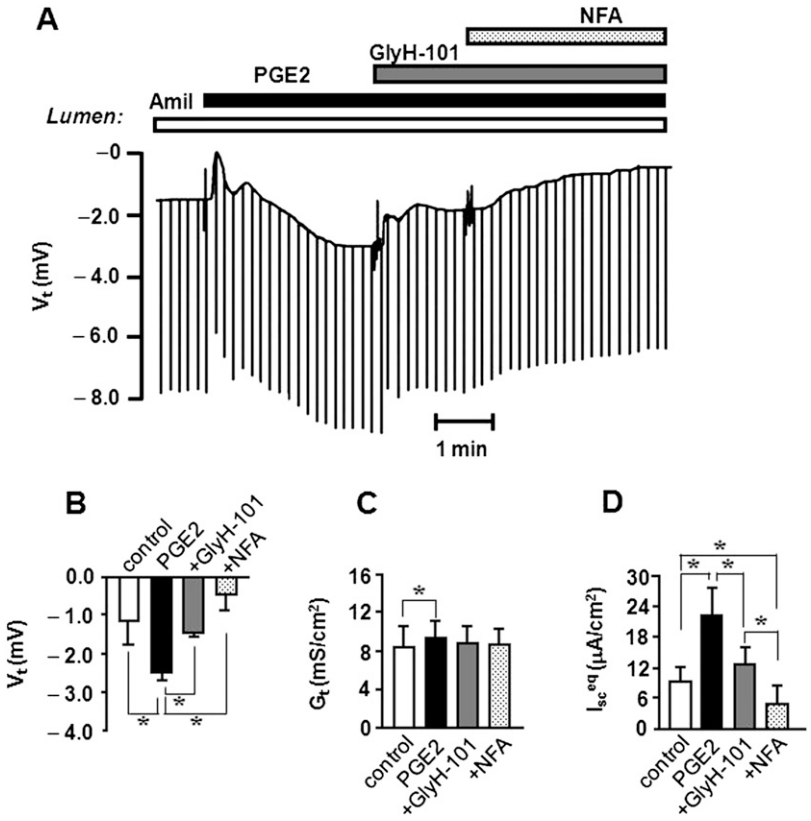

Figure 5. Effects of inhibitors, GlyH-101 and niflumic acid (NFA), on prostaglandin E (PGE) -2 stimulation. (*A*) Representative trace of electrogenic properties of HCO_3^- transport stimulated by luminal PGE2 (*black*; 1 μM). V_t hyperpolarized and I_{sc}^{eq} increased significantly ($P < 0.05$; $n = 4$). Luminal addition of inhibitor, GlyH-101 (*gray*), significantly decreased the HCO_3^- secretion, and further addition of the Ca^{2+}-activated chloride channel (CaCC) antagonist, NFA (*stippled*), further depressed the HCO_3^- secretion to levels significantly below the initial constitutive value. Summary of the effects of PGE2 (*black*), GlyH-101 (*gray*), and NFA (*stippled*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*). *$P < 0.05$; data presented are means ± SEM.

and vasoactive intestinal peptide. We observed no detectable effects of any of these agents (data not shown).

Discussion

Two clinical facts pointedly indicate that human small airways should be capable of secreting HCO_3^- physiologically. First, HCO_3^- secretion is severely depressed or absent in pathologically affected organs in CF in general (1, 10, 15). Second, most morbidity and mortality in CF are defined by failure in small airways (16–18). These fundamental observations encompass the additional facts that: (*1*) CFTR, the culprit protein in CF, is localized along the apical border of airway epithelia on ciliated cells (31, 33); and (*2*) CFTR supports transport of, and/or is permeable to, HCO_3^- (6, 8, 30).

Due to the small size, tedious dissectability, and tubular branching morphology of small airways, neither physiological regulation, nor even HCO_3^- secretion itself, has been demonstrated in native small airways. However, we recently introduced a miniaturized Ussing chamber (22) that permits very small specimens of epithelia (~ 1 mm ϕ) to be examined electrophysiologically, making it possible to evaluate the electrogenic signatures of electrolyte movements, including HCO_3^- transport. With this system, we used pig small airways (~ 1 mm ϕ) as a model for human tissue, especially because mutations for CF in transgenic pigs have recently become available experimentally (34, 35). To test this tissue for the presence of electrogenic HCO_3^--dependent properties, we first assayed for transepithelial HCO_3^- and Cl^- conductances using diffusion potentials from imposed chemical gradients. In the presence of a 150-mM HCO_3^- gradient, the V_t was approximately 70% of that of an equivalent Cl^- gradient (Figure 1), giving a selectivity ratio for Cl^-:HCO_3^- of approximately 5:1 (29), which compares favorably with approximately 4:1 of the CFTR single-channel selectivity ratio (6, 30), and seems consistent with the presence of this channel on the luminal surface of this epithelium (31). These results suggest that the CFTR channel conductance dominates the overall epithelial ion selectivity under these conditions, which would also include its expression in secretory as well as absorptive epithelial cells (22).

cAMP-Mediated HCO_3^- Secretion

Because mutations in CFTR are the basis of CF, and because CFTR is activated via increases in intracellular cAMP concentration (11, 36), we first tested the effects of directly elevating intracellular cAMP (with the adenylyl cyclase agonist, Fsk, combined with the phosphodiesterase inhibitor, IBMX). However, we observed the presence, even before applying these agents, of an unstimulated, constitutive I_{sc}^{eq} (mean = 10.6 ± 6.6; range over all experiments from ~ 5 to 20 μA/cm²). We found that Fsk/IBMX doubled the constitutive HCO_3^- I_{sc}^{eq}, which was effectively inhibited by the CFTR-selective inhibitor, GlyH-101 (32) (Figure 2, Table 1). These responses further illustrate the physiological presence of CFTR-dependent HCO_3^- secretion in the small airways. To test whether this secretion might involve physiological β-adrenergic regulation through G protein receptors, we applied the well known cAMP-mediated β-adrenergic agonist, IPR, and found that it also doubled the constitutive HCO_3^- I_{sc}^{eq}, which was also blocked by GlyH-101 (Figure 3, Table 1). These results strongly advocate for a CFTR-dependent pathway of HCO_3^- secretion in this tissue.

Unexpectedly, however, during these assays, we observed that adding inhibitors in addition to GlyH-101 to inhibit secretion by blocking HCO_3^- entry into the epithelial cells via possible Na^+-dependent HCO_3^- cotransporters and/or Cl^-/HCO_3^- exchangers, such as DIDS or DNDS (37, 38), reduced the HCO_3^- I_{sc}^{eq} to significantly less than the constitutive I_{sc}^{eq} (Figures 2 and 4). This result suggests the presence of another mechanism or pathway

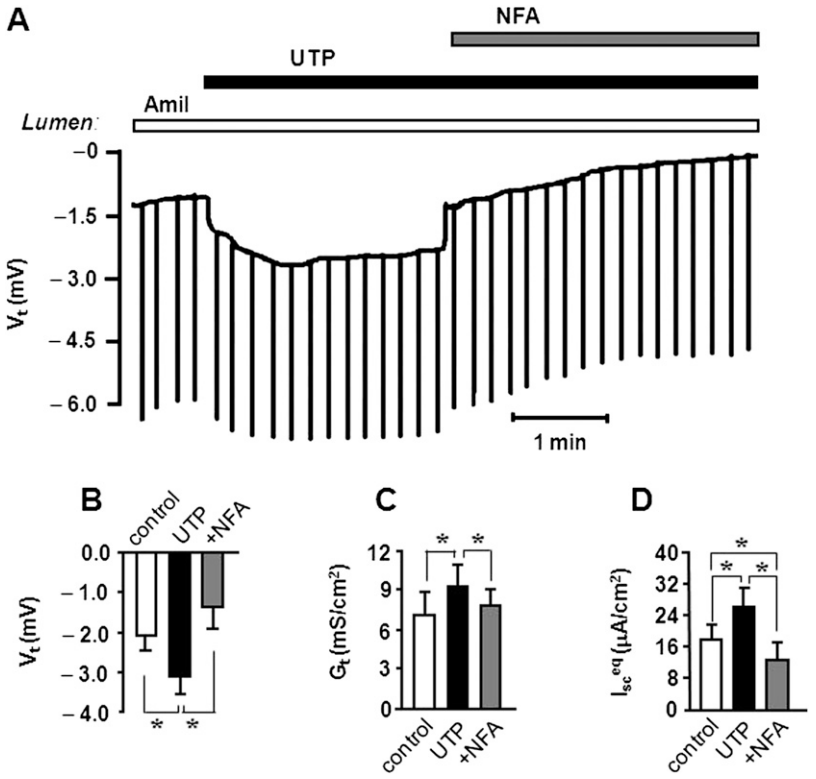

Figure 6. Effects of inhibitor, NFA, on UTP stimulation. (*A*) Representative trace of changes in electrical properties reflecting HCO_3^- transport induced by luminal addition of UTP (*black*; 100 μM), which significantly hyperpolarized V_t, increased G_t, and stimulated I_{sc}^{eq} ($P < 0.05$; $n = 5$). Luminal addition of the CACC inhibitor, NFA (*gray*), decreased the HCO_3^- secretion I_{sc}^{eq} to values significantly less than initial constitutive secretion in the continuous presence of luminal amiloride (*white*). Summary of the effects of UTP (*black*) and NFA (*gray*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*). *$P < 0.05$; data presented are means ± SEM.

for electrogenic HCO_3^- secretion in addition to CFTR-dependent transport. We cannot exclude the possibility that GlyH-101 conceivably did not completely block the CFTR-dependent path or that DIDS and DNDS may have had a nonspecific effect.

Ca^{2+}-Mediated HCO_3^- Secretion

We examined the possibility that another signaling pathway might be mediated from the luminal surface, possibly via Ca^{2+}-mediated purinergic receptors in the apical membrane (39–42). We tested the effect of adding the purinergic P2Y2 agonist, UTP, to the luminal bath, which, in fact, also significantly stimulated HCO_3^- I_{sc}^{eq} (Figure 6, Table 1). Applying luminal NFA to inhibit CaCC not only blocked the stimulated current, but also decreased the total current to significantly less than the constitutive I_{sc}^{eq} (Figure 6). Noting that PGE2 is a potent mucin secretagogue and may act via both cAMP and Ca^{2+} mediators (43, 44), we observed that, when we stimulated with this agonist, the I_{sc}^{eq} more than doubled, but GlyH-101 only partially blocked the increase (Figure 5); again, adding NFA to block possible CaCC-dependent activity (45, 46) further inhibited the HCO_3^- I_{sc}^{eq} to significantly below the initial constitutive current (Figure 5).

We surmised that, if HCO_3^- were secreted by separate components independently, concurrently stimulating the tissue with both cAMP- and Ca^{2+}-mediated agonists together might increase HCO_3^- I_{sc}^{eq} more than either agonist alone. That is, the responses might be additive, which appeared to be the case (Figure 7, Table 1). Taken together, these results support the notion of two separate pathways for HCO_3^- secretion in the small airways. We tentatively assume that a CFTR-dependent mechanism under β-adrenergic control is present, but independent of another mechanism possibly under purinergic control acting via CaCC-dependent secretion. Although CaCCs were not initially thought of as major HCO_3^- conductances, very good evidence has been presented for that role in oviduct tissue (47), and more recent evidence suggests that they may play such a role in goblet cells (48). The notion that Cl^- channels also physiologically transport HCO_3^- is not widely recognized, and has generally given way to a more classical view in which an apical Cl^-/HCO_3^- anion exchanger operates in parallel with a Cl^- conductance that recycles Cl^- effecting net HCO_3^- secretion (49). However, it does not seem possible that Cl^--dependent transport could be involved in our measurements, because Cl^- was absent from both apical and basolateral solutions. This conclusion does not in any way rule out the function and contribution of anion exchangers when Cl^- is present physiologically, which would only add to the tissue's capacity to transport HCO_3^-.

Why Two?

The suggestion of two independent mechanisms for HCO_3^- secretion begs the question of "why two"? Although only guided speculation is possible at this point, it is intriguing to note that two new roles for HCO_3^- have come to light very recently beyond its conventional role as the principal extracellular physiological buffer. First, HCO_3^- was discovered to be critical for the bactericidal properties of antimicrobial peptides (AMPs) (50). Moreover, Pezzulo and colleagues (51) reported recently that the relatively more acidic airway surface fluid (most likely due to the lack of HCO_3^-) in CF transgenic pig airways failed to kill bacteria as efficiently as in wild-type pigs. Second, secreted HCO_3^- was also discovered to be crucial for the normal release and maturation of gel-forming mucins (12–15). Needless to point out, both of these functions are inherent in and crucial for innate defense of airways. Where and how HCO_3^- is secreted for AMP activity has not been considered, and conceivably could be under partial or separate Ca^{2+}-mediated control (52). Likewise, the source of HCO_3^- for normal mucin maturation has not been firmly established either, but appears to be mainly via cAMP-mediated stimulation of enterocytes in the small intestine (53). Because UTP acts via purinergic receptors and IPR acts via adrenergic receptors, we question whether the former might be

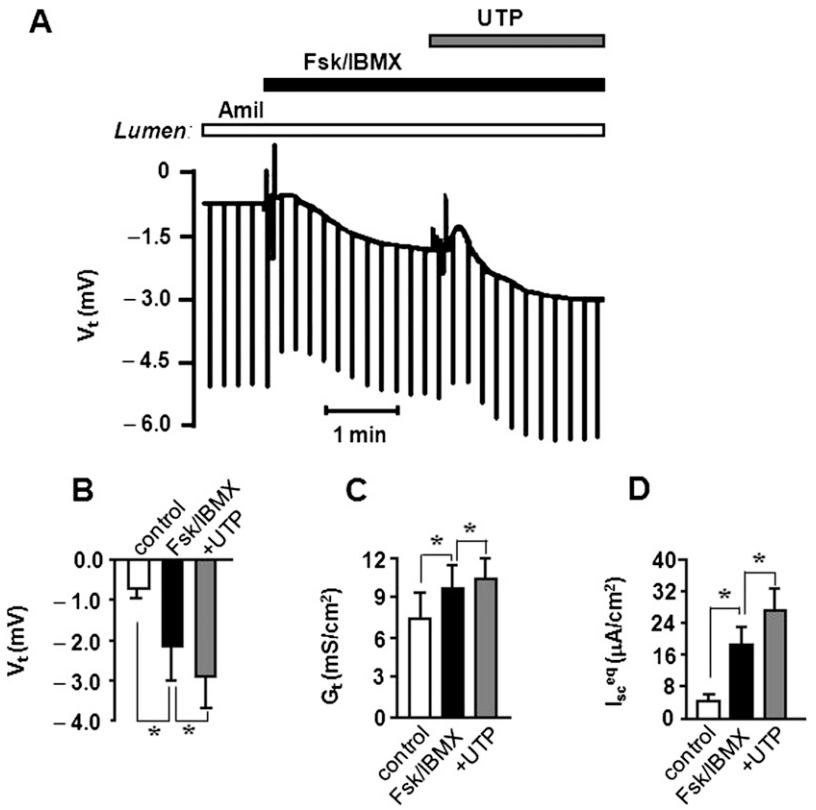

Figure 7. Additive effects of Fsk/IBMX and UTP stimulation. (*A*) Representative trace of electrical properties reflecting HCO_3^- transport when stimulated by Fsk/IBMX, which significantly hyperpolarized V_t, and increased G_t and I_{sc}^{eq} ($P < 0.05$; $n = 4$) in the presence of luminal amiloride. Subsequent addition of UTP to Fsk/IBMX hyperpolarized V_t further, and increased G_t and I_{sc}^{eq} even further ($P < 0.05$; $n = 4$). Summary of the additive effects of Fsk/IBMX (*black*) and UTP (*gray*) on V_t (*B*), G_t (*C*), and I_{sc}^{eq} (*D*) relative to control (*white*). *$P < 0.05$; data presented are means ± SEM.

associated more with AMP pathogen defense and the latter with mucin clearance defense.

The possibility of two independent HCO_3^- sources in the airways raises a propitious hope for potential pharmacotherapeutics for CF (54, 55) and possibly other diseases. That is, in CF for example, pharmacologically stimulating a Ca^{2+}-mediated source of HCO_3^- secretion independent of CFTR might enhance the effectiveness of AMP killing and/or significantly enhance a more normal discharge of goblet cell mucins and mucus in small airways.

Conclusions

In summary, native small airway epithelia appear to secrete HCO_3^- by at least two separate pathways. One pathway appears to be CFTR dependent and appears to be cAMP mediated via β-adrenergic receptors. The other pathway appears to be CaCC dependent, possibly via Ca^{2+}-mediated luminal purinergic receptors. These HCO_3^- secretions in the small airways would not only serve a conventional role as a pH buffer, but also critically support the maturation and discharge of mucins as well as the bactericidal activity of AMPs for innate defense. Defects in HCO_3^- secretion seem likely to predispose the innate defenses of small airways to pathogenic failure, as seen in CF. ■

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